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Low oxygen tension stimulates the redifferentiation of dedifferentiated adult human nasal chondrocytes[1]

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Summary

Objective: To determine the effect of dissolved oxygen tension (DO) on the redifferentiation of dedifferentiated adult human nasal septum chondrocytes cultured as pellets.

Design: After isolation, human nasal chondrocytes were expanded in monolayer culture, which resulted in their dedifferentiation. Dedifferentiated cells were pelleted, transferred to a bioreactor and maintained for up to 21 days at 100% DO (21% oxygen), 25% DO (5.25% oxygen) or 5% DO (1% oxygen), which was controlled in the liquid phase. Redifferentiation was assessed by staining the extracellular matrix with safranin-O and by the immunolocalization of collagen types I, II, IX and of a fibroblast membrane marker (11-fibrau). In addition, glycosaminoglycans (GAG) and DNA content were determined spectrophotometrically.

Results: In monolayer culture, cells dedifferentiated and multiplied 90- to 100-fold. Cell pellets cultured in a bioreactor under conditions of low oxygen tension (25% DO or 5% DO) stained intensely for GAGs and for collagen type II, but very weakly for collagen type I. After 14 days of culturing, cell pellets maintained at 5% DO stained more intensely for collagen IX and more weakly for 11-fibrau than did those incubated at 25% DO. After 21 days of culturing the GAG content of cell pellets maintained at 5% DO was significantly greater than that of those incubated at 25% DO.

Under air-saturated conditions (100% DO), the DNA and GAG contents of cell pellets decreased with time in culture. After 21 days of culturing, both parameters were substantially lower in cell pellets maintained at 100% DO than in those incubated at low oxygen tensions. The staining signals for collagen types II and IX were much weaker, and those for the markers of dedifferentiation (collagen type I and 11-fibrau) much stronger under air-saturated conditions than at low oxygen tensions.

Conclusion: These observations demonstrate that using the present set-up, low oxygen tension stimulates the redifferentiation of dedifferentiated adult human nasal chondrocytes in pellet cultures.

Key words: Oxygen tension, nasal septum chondrocytes, redifferentiation, dedifferentiation.

Introduction

The treatment of articular cartilage defects is a challenge for orthopaedic surgeons worldwide. As early as 1743 it was known that these defects do not heal spontaneously[1]. Moreover, if a cartilage defect is left untreated, it will not only fail to heal, but will enlarge with time[2,3] and thus leading to further tissue degeneration[4]. Clinical intervention is thus inevitable. Since cartilage is a relatively simple tissue, which is composed of only one cell type, tissue engineering is deemed to be an approach to the treatment of articular cartilage defects[5].

For the repair of cartilage, an autologous treatment strategy is preferred, owing to the risks of triggering an immunogenic response[6] and of disease transfer[7] that are associated with allogenic approaches. As a cell source, articular cartilage from low weight-bearing regions could be used, but, due to donor site morbidity, the amount of tissue that can be obtained is small. Hence, the use of alternative autologous cell sources, such as human septum cartilage, have been evaluated[8,9]. In order to reduce the size of the biopsy removed, cells must be expanded *in vitro* to obtain a sufficient number for seeding of a porous scaffold. During expansion, the chondrocytes tend to dedifferentiate, which entails the loss of their spherical shape and the acquisition of a fibroblast-like appearance[10–12]. Accordingly, expression of the hyaline cartilage markers aggrecan and collagen type II decreases, whereas that of the non-hyaline-cartilage-specific collagen type I increases[11].

Previous investigations have evaluated role of *in vivo* factors in stimulating the redifferentiation of dedifferentiated chondrocytes *in vitro*. Within healthy articular cartilage, the oxygen concentration is typically low, with a tension of around 1% [=5% dissolved oxygen (DO)][13]. Oxygen is an essential nutrient, which plays a key role as an electron acceptor in the mitochondrial respiratory chain and directs several developmental processes, including chondrogenesis[14–16]. Chondrocytes embedded within

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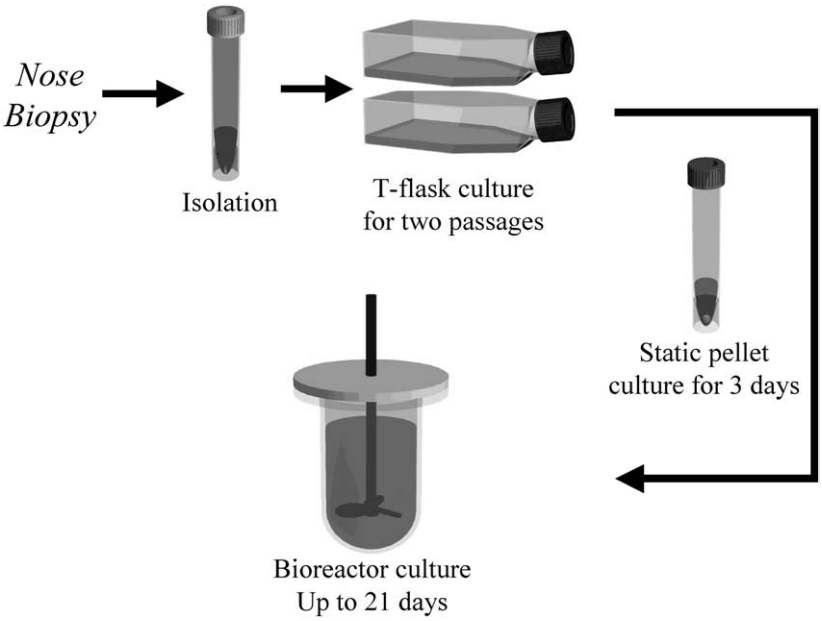

Fig. 1. Experimental design. Human nasal chondrocytes were isolated and expanded for 12 days in monolayer culture (two passages). To evaluate the post-expansion chondrocytic phenotype, the resulting cells were seeded in pellets and left for 3 days to form solid aggregates. Pellets were then transferred to a controlled bioreactor and cultured dynamically for up to 21 days.

cartilage adjust their metabolism to the low oxygen tension[17–19]. Hence, low oxygen tension has been investigated as a specific stimulus for chondrocyte redifferentiation. However, existing reports yield conflicting data[14].

Low oxygen tension (5–25% DO) has been demonstrated by several authors to stimulate chondrocyte redifferentiation[15,16,20–22]. But other investigators have reported the expression of differentiation markers, such as proteoglycan production, to be higher under more aerobic (100% DO) conditions[23–26] than at low oxygen tensions (5% DO). Yet other studies have demonstrated no difference in the production of proteoglycans at oxygen tensions between 5% and 100%[27–29]. Nevertheless, hyperoxic conditions (>100% DO) have been consistently reported to be unfavorable to the formation of for cartilage-like tissue[23,30,31].

In the previous investigations, oxygen tension was controlled in the gas phase. This renders a comparison of results difficult, since different experimental set-ups may significantly influence the ultimate concentration of oxygen in the liquid phase[32]. Hence, in the present study, we measured and controlled the oxygen concentration in the liquid phase. So doing, we evaluated the effect of different oxygen tensions on the redifferentiation of dedifferentiated adult human nasal septum chondrocytes. Isolated chondrocytes were expanded *in vitro* and the pelleted cells were then dynamically cultured in controlled bioreactors (Fig. 1). Cultures were maintained at 100%, 25% or 5% DO (21%, 5.25% and 1% oxygen, respectively). Redifferentiation was assessed by monitoring glycosaminoglycans (GAG) production and by the immunolocalization of collagen types I, II, and IX and of 11-fibrau.

Materials and methods

CELL ISOLATION

Nasal cartilage was obtained from patients [n=3 (age range: 28–71 years)] undergoing nasal septum reconstruction. The data reported herein relate to a biopsy from one patient only (age: 28 years), since we succeeded in isolating sufficient cells from this material to perform experiments at each of the three different oxygen concentrations. Cartilage was cut into 1-mm^3 blocks and the chondrocytes were isolated by digestion overnight in 0.15% type II collagenase (Worthington Biochemical). After three washes in phosphate buffered saline, cells were suspended in expansion medium [HEPES-buffered DMEM-Glutamax-1 (Gibco-BRL) supplemented with 10% foetal calf serum (Sigma-Aldrich), 50 µg ml^{-1} ascorbic acid 2-phosphate (Gibco-BRL), 10 ng ml^{-1} bFGF (Instruchemie), 100 units ml^{-1} penicillin (Gibco-BRL) and 100 µg ml^{-1} streptomycin (Gibco-BRL)].

CHONDROCYTE PROLIFERATION AND HARVESTING

Isolated chondrocytes, suspended in expansion medium, were plated within 300-cm^2 culture flasks at 3500 cells cm^{-2} and maintained at 37°C in a humidified 5% CO_2 incubator for two passages. When cultures reached subconfluence, chondrocytes were detached from flasks with 0.25% trypsin/1.0 mM EDTA (Sigma-Aldridge) and counted using a particle count and size analyzer (Coulter Corporation). Cell viability was assessed in a hemocytometer using the trypan-blue (Sigma-Aldridge) exclusion test.

PELLET PREPARATION AND CULTURING

Five hundred thousand cells in suspension were transferred to a 12-ml polypropylene centrifuge tube and centrifuged for 2 minutes at 500×g[33]. The resulting pellets were cultured within the tubes in redifferentiation medium[8] [DMEM-Glutamax-1 supplemented with 1% non-essential amino acids, 100 units ml^{-1} penicillin, 100 µg ml^{-1} streptomycin, 10% foetal calf serum, 10 µg ml^{-1} insulin (Sigma-Aldrich) and 50 µg·ml^{-1} ascorbic acid] for 3 days at 37°C in

a humidified 5% CO_2 incubator. Subsequently, 100 chondrocyte pellets (each containing 5×10^7 cells) per condition were transferred to a controlled 1l bioreactor (Applikon Dependable Instruments) with a working volume of 300 ml. The oxygen concentration within the agitated (marine impeller: 125 rpm) liquid phase was controlled using an oxygen electrode (Applikon Dependable Instruments) in combination with a computerized control system (Applikon Dependable Instruments) and headspace aeration with a mixture of nitrogen, oxygen and carbon dioxide. The pH was measured with a pH probe (Applikon Dependable Instruments) and maintained at 7.2 by varying the carbon dioxide concentration in the headspace. Pellets were cultured for up to 21 days at 37°C at 100% DO (21% oxygen), 25% DO (5.25% oxygen) or 5% DO (1% oxygen). Twice a week, half of the redifferentiation medium was refreshed. Samples were withdrawn after 7, 14 and 21 days of culturing in the bioreactor.

HISTOLOGICAL ANALYSIS

Cell pellets (3 per condition per time point) were withdrawn from the bioreactor after 7, 14 and 21 days of culturing and fixed in 0.14 M cacodylate buffer (pH= 7.2–7.4)/1.5% glutaraldehyde (Merck). They were then dehydrated, embedded in glycol methacrylate (Merck). 5 µm-thick sections were prepared. Cells were stained with hematoxylin (Sigma-Aldrich) and fast green (Merck) and GAGs with safranin-O (Sigma-Aldrich). Samples were observed in a light microscope (E600, Nikon).

IMMUNOHISTOCHEMISTRY

Cell pellets (3 per condition per time point) were withdrawn from the bioreactors after 7, 14 and 21 days of culturing and embedded in optimal-cutting-temperature compound (Tissue-Tek). 5 µm thick cryosections were prepared and fixed in acetone for 3 min. Sections were immunostained for collagen types I, II and IX and for 11-fibrau.

IMMUNOSTAINING FOR COLLAGENS

Sections were incubated for 1 h in protein-blocking buffer (Dako). Those destined for collagen-type-IX staining were digested with 1% pepsin (pH=3) for 30 min at 37°C. Sections were treated with monoclonal antibodies against collagen types I [Ab-1 (Calbiochem), diluted 1: 100], II [II-II6B3 (Developmental Studies Hybridoma Bank), diluted 1:200] or IX [B3-1 (Developmental Studies Hybridoma Bank), diluted 1:100] for 1 h. After rinsing with PBS containing 10% human serum, sections were incubated with a secondary horse-radish-peroxidase-conjugated goat anti-mouse antibody [(DAKO) diluted 1:100] for 1 h. Immunoreactivity was visualized by incubating sections with DAB-solution (DAKO) for 10 min. Sections were counterstained with hematoxilin (Sigma).

IMMUNOSTAINING FOR 11-FIBRAU

The monoclonal antibody 11-fibrau binds to a 112-kD protein on the cell surface of fibroblasts. This protein is absent from differentiated chondrocytes, but is expressed by dedifferentiated ones[34]. Sections were incubated first with blocking buffer (Boehringer) containing 10% normal goat serum, then with the 11-fibrau antibody [D7-fib (ITK Diagnostics BV), diluted 1:400] for 2 h, next with alkaline-phosphatase-conjugated anti-mouse Fab-fragments [GAMAP (Immunotech), diluted 1:100] for 30 min and finally with a monoclonal antibody against mouse alkaline phosphate anti-alkaline phosphatase [(Dakopatts) diluted 1:100], also for 30 min. Immunoreactivity was visualized by incubating sections with a new fuchsin substrate (Chroma), which demonstrates alkaline phosphatase activity (red staining).

Controls for all staining procedures were prepared by following the routine protocol in the absence of the first antibody.

BIOCHEMICAL ANALYSIS

Pellets were pooled [(4 per condition per time-point (N=3)] and digested overnight at 56°C in a solution containing proteinase K, pepstatin A and iodoacetiamide (Sigma-Aldrich). Total DNA was quantified spectrofotometrically (Perkin Elmer) using the Cyquant dye kit (Molecular Probes). GAGs were likewise determined spectrofotometrically after reaction with dimethylmethylene blue [DMMB (Sigma-Aldrich)][35]. Color intensity was measured immediately in a microplate reader [EL 312e (Bio-TEK Instruments)] at an absorption wavelength of 520 nm. The quantity of GAGs was determined using a standard concentration of chondrotin sulphate B (Sigma-Aldrich).

STATISTICS

Statistical significance (P<0.05) was assessed by an analysis of variance (ANOVA) and by Tukey's posthoc test using Sigma Stat (SPSS Inc.).

Results

MONOLAYER CULTURES

Isolated cells attached to the surface of polystyrene flasks and gradually spread during the first 3 days of culturing (Fig. 2A). These spindle-shaped, fibroblast-like cells proliferated and reached sub-confluence within 7 days (Fig. 2B). After sub-culturing, cells attached more rapidly and reached sub-confluence within 5 days. At the time of harvesting, cell number had increased an approximately 90- to 100-fold. According to the trypan-blue exclusion test, cells were viable at this juncture (data not presented).

During monolayer culture, a switch in collagen production from type II to type I was observed. After 12 days, cells manifested signs of dedifferentiation. At this juncture, they no longer produced either collagen type II (Fig. 3B) or collagen type IX (Fig. 3C), but collagen type I was synthesized in abundance (Fig. 3A).

REDIFFERENTIATION IN PELLET CULTURES

Dynamic culturing of cell pellets under air-saturated conditions (100% DO) for 7 days yielded weak staining for GAGs (Fig. 4A). The staining intensity did not further increase with time (Fig. 4B, 4C). During the entire culturing period, cells had a fibroblast-like morphology. After 21 days, a substantial reduction in pellet size was observed (Fig. 4C).

After 7 days, cell pellets cultured at 25% DO were larger, than those cultured at either 100% DO or 5% DO (Fig. 4D). Intense staining for GAGs was observed, which was most

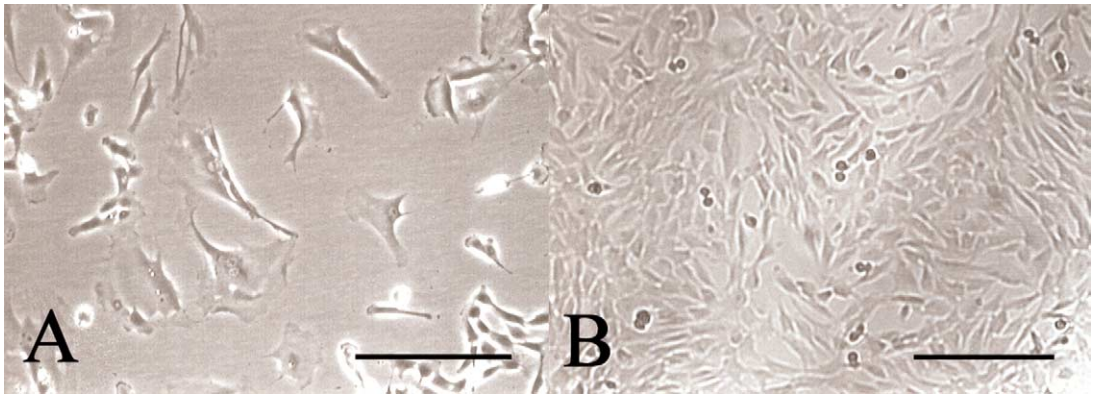

Fig. 2. Photomicrographs of human septum nasal chondrocytes attached to the surfaces of polystyrene flasks after 3 days (A) and 7 days (B) of culturing. Scale bars represent 100 μm (A) and 150 μm (B).

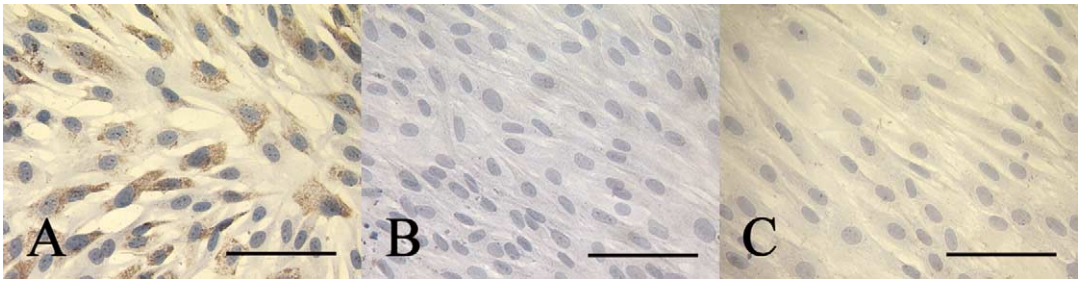

Fig. 3. Photomicrographs of immunolocalized collagen types I (A), II (B) and IX (C) in monolayer cultures (first passage) after 12 days of cell expansion. Scale bars represent 100 μm.

prominent near the periphery of aggregates. GAG staining intensely increased with time (Fig. 4E, 4F), after 21 days, the tissue had a hyaline-like cartilage appearance (Fig. 4F).

After 7 days, cell pellets cultured at 5% DO stained only weakly for GAGs (Fig. 4G). Staining intensity increased between 14 and 21 days (Fig. 4H, 4I), and at the latter juncture (Fig. 4I) was comparable to that observed for cell pellets cultured at 25% DO for a similar period (Fig. 4F). Cells had a rounded appearance and were surrounded by an intensely-stained extra cellular matrix (Fig. 4I).

The decrease in pellet size observed after culturing at 100% DO correlated with a significant decrease in DNA content (Fig. 5A). The DNA content of pellets maintained at 25% DO did not change significantly during culture, whereas that of pellets incubated at 5% DO increased (Fig. 5A). The DNA content of pellets cultured at 25% DO and 5% DO did not differ significantly from each other.

GAG content assessed quantitatively by the DMMB assay correlated with the intensity of safranin-O staining determined qualitatively(Figs. 4 and 5B). Pellets cultured at 100% DO contained smaller amounts of GAGs than did those maintained at either 25% DO or 5% DO. The reduction in pellet size correlated with the decrease in GAG content. After 21 days culturing, pellets maintained at 5% DO contained 23.7±1.7 μg of GAGs per pellet, which was significantly greater than the amount found within pellets incubated at 25% DO (18.7±0.7 μg of GAGs per pellet). Interestingly, after 14 days of culturing, pellets maintained at 25% DO had a significantly higher GAG content than did those incubated at 5% DO.

Pellets dynamically cultured for 14 days at 100% DO stained intensely for the fibroblast marker 11-fibrau (Fig. 6A). For pellets cultured at 25% DO, staining was less strong and associated mainly with the outer layer of fibroblast–like cells covering the pellet (Fig. 6B). Culturing at 5% DO yielded weak staining for 11-fibrau, which was confined to the outer margins of the aggregates (Fig. 6C). After 14 days of culturing, collagen type IX was observed only within pellets incubated at 25% or 5% DO (Fig. 6E, 6F), not in those maintained at 100% DO (Fig. 6D).

Collagen type I was still present within in all pellets dynamically cultured for 21 days (Fig. 7A, 7B, 7C). However, in pellets cultured at lower oxygen tensions, the pattern of immunostaining was less homogeneously distributed, indicating a lower level of collagen type I production. After 21 days of culturing, staining for collagen type II was more abundant in pellets incubated at 25% DO and 5% DO, than in those maintained at 100% DO (Fig. 7D, 7E, 7F). Immunostaining for collagen type II was most intense and most homogeneously distributed within pellets cultured at 25% DO for 21 days.

Discussion

In the present study, we evaluated the effect of reduced oxygen tension on the redifferentiation of dedifferentiated adult human chondrocytes derived from the nasal septum. After expansion in monolayer cultures, cell aggregates were dynamically cultured in a controlled bioreactor at air-saturated (100% DO) and reduced oxygen tensions (25% and 5% DO).

During expansion in monolayer cultures, the nasal chondrocytes underwent dedifferentiation. Changes observed during this period of expansion included a switch to a fibroblast-like morphology and a switch in collagen

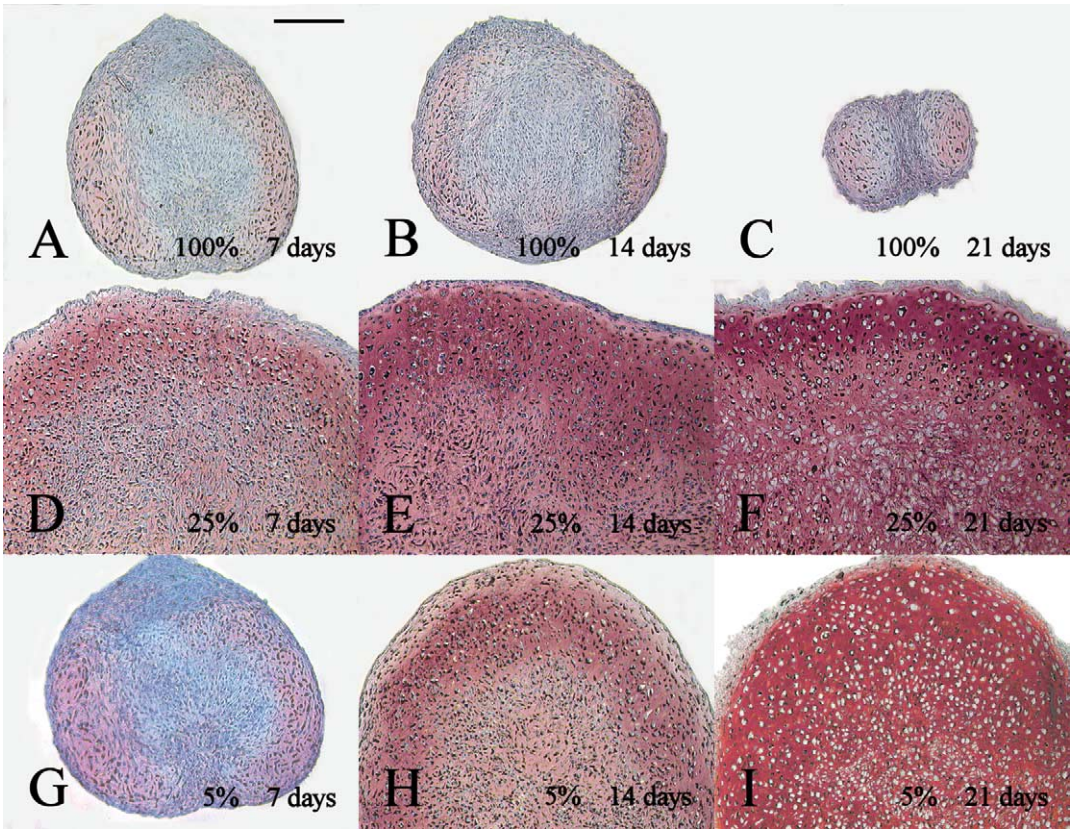

Fig. 4. Photomicrographs of safranin-O stained sections derived from dynamically cultured cell pellets after 7 days (A, D, G), 14 days (B, E, H) and 21 days (C, F, I) of culturing at 100% DO (A, B, C), 25% DO (D, E, F) and 5% DO (G, H, I). Scale bar represents 250 μm.

synthesis from types IX and II to type I. These are typical features of dedifferentiation and have been widely described for articular chondrocytes[11,12,36,37].

Dedifferentiated cells were pelleted and dynamically cultured in a bioreactor under controlled conditions of oxygen tension, which was monitored within the liquid phase. Dynamic culturing under air-saturated conditions resulted in a gradual decrease in pellet size, in GAG content and in the production of collagen types II and IX. In contrast the synthesis of 11-fibrau (a 112-kD protein), which is typically expressed by fibroblasts[34,38], and of collagen type I increased. Hence, small pellets of cells cultured under air-saturated conditions, maintain their dedifferentiated phenotype.

On the other hand, pelleted cells cultured at 25% DO began to produce GAGs within 7 days. Some staining for 11-fibrau was still observed after 14 days, but no collagen type I could be detected after 21 days. However collagens type IX and II were abundantly expressed after 14 and 21 days, respectively. The onset of GAG production in cultures maintained at 5% DO was not as rapid as within those incubated at 25% DO. However, after 21 days, pellets maintained at 5% DO had a significantly higher GAG content. They exhibited weak staining for collagen type I and for 11-fibrau at this stage, whereas collagen types II and IX were present in abundance throughout the whole pellet. These results are consistent with previous findings relating to the stimulatory effects of low oxygen tension on GAG synthesis in embryonic chick chondrocytes[16,39], and in bovine chondrocytes cultured either in monolayers[22] or encapsulated within alginate beads[20,21]. However, they do not accord with the findings of several other studies, which report enhanced redifferentiation at more aerobic oxygen levels within cartilage explants[23], monolayer cultures[25] and tissue-engineered cartilage constructs[26]. Using the experimental set-up described in the present study, we could control and monitor the oxygen tension experienced by cell pellets in the agitated liquid phase. In the above-cited studies using cartilage explants and chondrocyte monolayer cultures, oxygen tension was controlled and measured within the gas phase. Due to diffusional constraints, the oxygen concentration at the cellular level is likely to be lower when thus monitored. Obradovic *et al.*[26] cultured tissue-engineered cartilage constructs in a rotating bioreactor at 100% DO. They demonstrated that, depending on gaseous exchange, oxygen concentrations within the liquid phase differed considerably from those within the surrounding gas phase. Nevertheless, they reported higher concentrations of oxygen in the liquid phase (54% DO) to be more favorable to the formation of cartilage-like tissue than lower ones. This finding could reflect the size of the cultured constructs used (5×2 mm), which was substantially larger then our cell pellets (approximately 1 mm in diameter). Recently, microelectrode[40] and fluorescence-sensor[41] measurements have confirmed the existence of oxygen gradients within 3-dimensional tissue-engineered cartilage constructs. Oxygen concentration will decrease rapidly with increasing distance from the bathing medium, and the level experienced by most cells will thus be lower than that within the

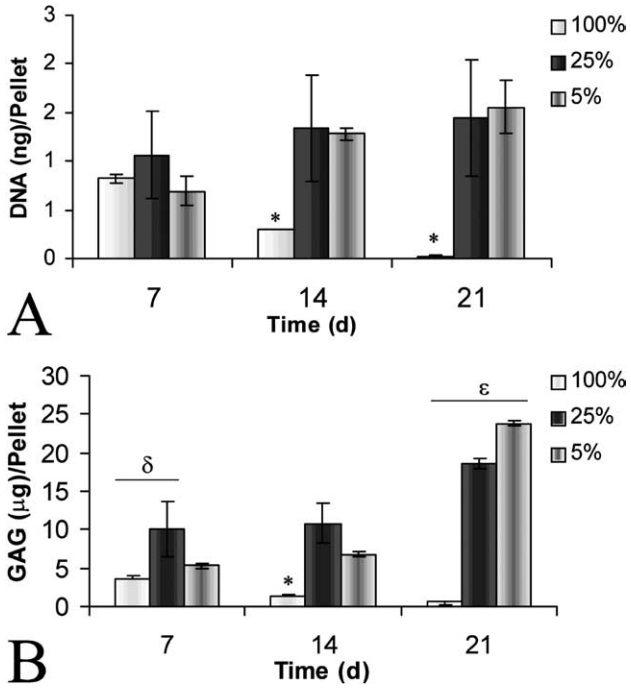

Fig. 5. Graphs depicting the DNA (A) and GAG contents of cell pellets after 7 days, 14 days and 21 days of culturing at 100% DO, 25% DO or 5% DO. Mean values (±SD) are represented. At each sampling time, values for a particular DO tension that differ significantly ($P<0.05$) from the other two are indicated by an asterisk. Other significant differences are indicated by the symbols 'δ' and 'ε'.

surrounding agitated liquid phase. Consequently, the oxygen concentrations reported to be optimal for the formation of cartilage-like tissue could be higher than the optimal concentration at the cellular level.

Recent investigations performed by Domm *et al.*[20,42] and by Murphy and Sambanis[21] have demonstrated that passaged chondrocytes incorporated into alginate express higher levels of collagen type II when the oxygen concentration in the incubators is lowered to 24% DO (5% oxygen). As explained above, the oxygen concentration experienced by the cells is likely to have been lower due to oxygen transport resistance at the gas-liquid interface and within the alginate beads.

In contrast to our results and the data reported by Domm *et al.*[20,42] and by Murphy and Sambanis[21], Grimshaw and Mason[27] reported a down-regulation of collagen type II for bovine chondrocytes cultured at 24% DO. However, in the present investigation, as well as in the previously reported studies[20,21], the redifferentiation of dedifferentiated cells was evaluated over several weeks, whereas Grimshaw and Mason assessed this process for primary chondrocytes over the course of only 7 days.

With the experimental set-up used in the present study, we can control the oxygen tension experienced by cell pellets in the agitated liquid phase. Nevertheless, as aforementioned, oxygen concentration will decrease with increasing distance from the pellet periphery, and gradients will thus be generated[41,43]. In the future, these gradients could be predicted using mathematical models[40] or measured with microelectrodes[44].

Only selected analyses were performed on samples derived from the two other patients (aged 47 and 71 years). On the basis of enhanced GAG staining at 5% DO compared with that at 100% DO, the results of the 47-year-old patient support the concept of a stimulatory effect of low oxygen tension on the redifferentiation of dedifferentiated. In samples derived from the 71-year-old patient, GAG staining within pellets cultured at 25% DO and 100% DO were similar, suggesting that the effects of low oxygen tension may be age-dependent. However this aspect needs to be addressed systematically in future investigations. Whether culturing at 25% DO or at 5% DO is most advantageous must now be established using smaller gradations in oxygen tension.

In summary, our findings demonstrate that the differentiation state of human nasal chondrocytes can be

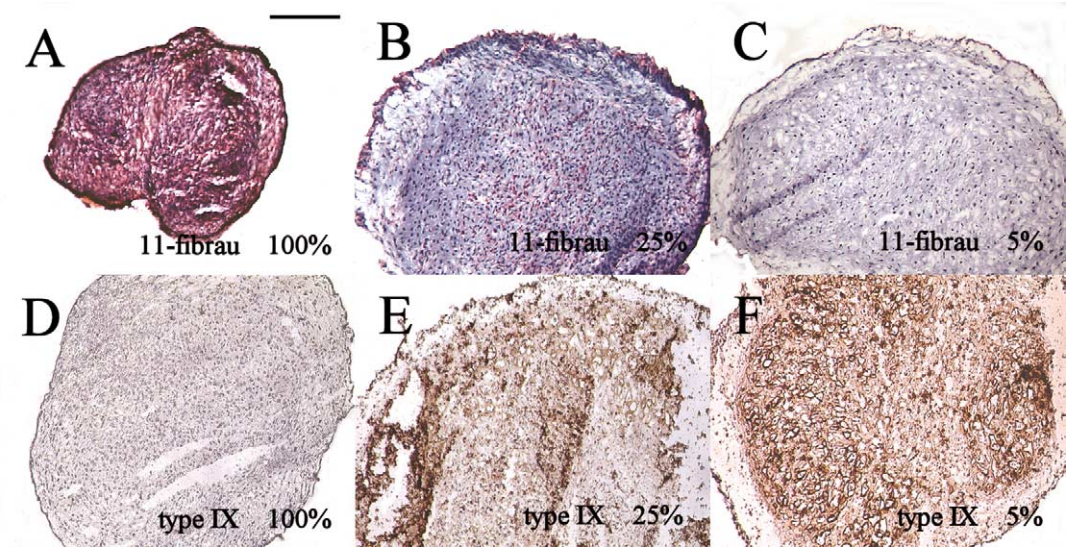

Fig. 6. Photomicrographs of immunolocalized 11-fibrau (A, B, C) and collagen type IX (D, E, F) on sections of cell pellets cultured dynamically for 14 days at 100% DO (A, D), 25% DO (B, E) or 5% DO (C, F). Scale bar represents 250 μm.

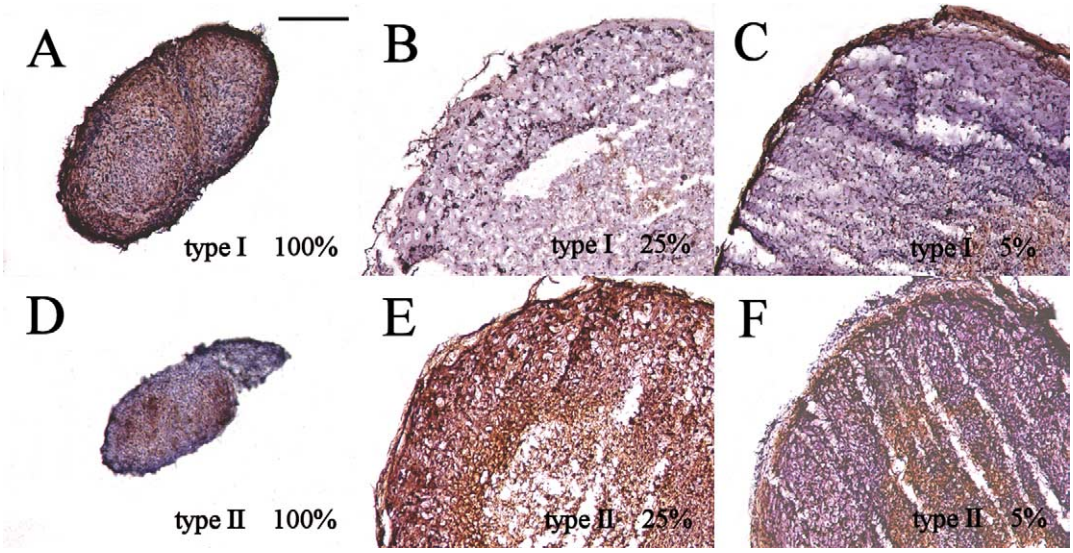

Fig. 7. Photomicrographs of immunolocalized collagen types I (A, B, C) and II (D, E, F) on sections of cell pellets cultured dynamically for 21 days at 100% DO (A, D), 25% DO (B, E) and 5% DO (C, F). Scale bar represents 250 μm.

modulated by changes in oxygen tension. Pellet cultures maintained for 21 days at low oxygen tension (25% DO or 5% DO) had a higher GAG and cell number (DNA content) and stained more intensely for collagen type IX, than did those incubated under air-saturated conditions (100% DO). Immunoreactivity for collagen type II was stronger and that for collagen type I weaker at 25% DO than at 5% DO.

These findings confirm that by mimicking the low oxygen tension present within cartilage *in vivo*, dedifferentiated chondrocytes can be stimulated to redifferentiate *in vitro*. The oxygen tension conditions established during the culturing of tissue-engineered cartilage constructs should thus be carefully considered.

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